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Investigation on the development, diagnosis and therapy of ketosis in non-gravid and non-lactating guinea pigs

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Vetsuisse-Fakultät Universität Zürich (2018)
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Investigation on the development, diagnosis and therapy of ketosis in non-gravid and non-lactating guinea pigs

Because of the clinical relevance of ketosis in guinea pigs (*Cavia porcellus*), we performed a feeding experiment with 28 adult males and females of obese and slim body condition. Animals were fasted for 3 days and refed afterwards; half of them received a supportive glucose therapy. Ketone bodies were measured in serum and urine during and after fasting. ALT, bile acids and liver histology were analyzed after 7 days of refeeding (and therapy). Obese guinea pigs, and females, showed a significantly higher increase in ketone bodies in serum and urine. Obese, female, and non-treated animals needed more time to regulate ketone bodies to normal levels. Liver histology revealed increased hepatocyte degeneration and higher glycogen content in obese and therapied animals, and additionally more glycogen in males. Only minor hepatic fat accumulation occurred. Bile acids showed good correlation to histological liver changes whereas ALT did not. Female and obese animals react more intensively to fasting. As preventive management, animals should be kept in adequate body condition, fasting should be avoided, and anorexia should be treated immediately. In such a case, urinary dip stick to detect ketone bodies is a useful diagnostic tool. Glucose therapy leads to faster cessation of ketogenesis and we recommend it in cases of ketosis. However, it needs to be adjusted to avoid hepatocyte glycogen overload and degeneration. Measuring bile acids presents a valuable indicator of liver damage.

Key words: Guinea pig, Fasting ketosis, Beta-Hydroxybutyrate, Bile acid, Liver damage

Vetsuisse-Fakultät Universität Zürich (2018)
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Untersuchungen zur Entstehung, Diagnostik und Therapie der Ketose bei nicht tragenden und nicht säugenden Meerschweinchen

Die Ketose beim Meerschweinchen (*Cavia porcellus*) stellt eine klinisch relevante Erkrankung dar, weshalb wir ein Experiment mit 28 adulten männlichen und weiblichen Meerschweinchen in schlankem und obesem Nährzustand durchführten. Die Tiere wurden 3 Tage gefastet und danach angefüttert; die Hälfte erhielt unterstützende Glukosetherapie. Während und nach dem Fasten wurden Ketonkörper in Urin und Serum gemessen. ALT, Gallensäuren und Leberhistologie wurden 7 Tage nach Anfüttern analysiert. Obese und weibliche Tiere zeigten einen höheren Anstieg der Ketonkörper in Serum und Urin. Obese, Weibchen und nicht therapierte Tiere hatten längere Eliminationsphasen der Ketonkörper. Die Hepatozyten der obesen und therapierten Tiere wiesen mehr Degeneration und Glykogen auf, letzteres auch bei den Männchen. Insgesamt sahen wir nur wenig Fettakkumulation. Gallensäuren haben eine gute Korrelation mit histologischen Leberveränderungen, im Gegensatz zur ALT. Weibliche und obese Tiere reagieren stärker auf einen Fastenzustand. Präventiv sollte der Nährzustand adäquat gehalten und Fasten vermieden werden. Bei anorektischem Zustand muss rasch gehandelt werden. Dabei sind die Combursticks wertvoll für die Diagnostik. Für einen schnellen Rückgang der Ketogenese wird Glukosetherapie empfohlen. Die Menge sollte angepasst werden, um einen Glykogen-Überschuss und folglich Hepatozyten-Degeneration zu verhindern. Als Indikator einer Leberschädigung sind Gallensäuren wertvoll.

Schlüsselwörter: Meerschweinchen, Fastenketose, Beta-Hydroxybutyrat, Gallensäuren, Leberschaden

Investigation on the development, diagnosis and therapy of ketosis in non-gravid and non-lactating guinea pigs

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***Abstract*:**
Objective: To observe progression of ketosis in guinea pigs, document changes and evaluate a therapeutic approach.
Animals: 28 adult guinea pigs (*Cavia porcellus*), male and female of obese and slim body condition.
Procedures: Animals were fasted for 3 days and refed afterwards; half of them received a supportive therapy with glucose subcutaneously. Ketone bodies were measured in serum and urine during and after the fasting. ALT, bile acids and liver histology were analyzed after 7 days of refeeding (and therapy).
Results: Obese guinea pigs, and females, showed a significantly higher increase in ketone bodies in serum and urine. Obese, female, and non-treated animals needed more time to regulate ketone bodies to normal levels. Liver histology revealed increased hepatocyte degeneration and higher glycogen content in obese and therapied animals, and additionally more glycogen in males. Only minor hepatic fat accumulation was documented. Bile acids showed good correlation to histological liver changes whereas ALT did not.
Conclusion and Clinical Relevance: Female and obese animals react more intensively to fasting. As preventive management, animals should be kept in adequate body condition, fasting should be avoided, and anorexia should be treated immediately. In such a case, urinary dip stick to detect ketone bodies is a useful diagnostic tool. Glucose therapy leads to faster cessation of ketogenesis and we recommend it in cases of ketosis. However, it needs to be adjusted to avoid hepatocyte glycogen overload and degeneration. Measuring bile acids presents a valuable indicator of liver damage.

Keywords: Guinea pig, Fasting ketosis, Beta-Hydroxybutyrate, Bile acid, Liver damage

Abbreviations

ALT	Alanine Aminotransferase
BHB	β-Hydroxybutyrate
GLM	General linear model
IFCC	The International Federation of Clinical Chemistry and Laboratory Medicine
POC	Point of care

1 Introduction

The guinea pig (*Cavia porcellus*) is one of the most frequently presented small pets at clinics [1,2]. Guinea pigs often show unspecific signs of depression and inappetence, mostly as a result of an underlying disease. In case of delayed or absent treatment, there is a risk of additional metabolic disorders. One important metabolic disorder is ketosis, often encountered in combination with fatty liver disease, caused by anorexia after a period of feeding on a high energy diet [3,4]. Ketosis, by definition, is an accumulation of acetoacetate, β-hydroxybutyrate (BHB) and acetone in body fluids as a result of increased fat mobilisation for energy production from lipids. Due to an excessive beta-oxidation of fatty acids, more acetyl-coenzyme-A is synthetized than can be used for gluconeogenesis; this leads to an accelerated ketogenesis in hepatocytes [5].

Ketosis is described in various animal species from cattle, sheep and goats to rabbits, hamsters, guinea pigs and nonhuman primates as well as humans [6,7]. Multiple studies reported a variety of clinical signs in relation with ketosis when fasting guinea pigs after a period of feeding an energy-dense diet for different amounts of time. In most cases, pregnant guinea pigs were investigated and a moderate to severe clinical manifestation of ketosis in obese patients could be induced, while in non-pregnant guinea pigs and males, none to subclinical changes were observed more often [4,6,8,9]. Ketosis of pregnant guinea pigs is also referred to as 'pregnancy toxicosis' [10,11]. Predisposing factors are obesity, lack of exercise, large fetal loads and primiparity, a change in diet or environment, heat stress, and possibly hereditary susceptibility [10,12]. Lachmann, et al. [4] wrote that the syndrome of ketosis can be triggered primarily by anorexia and is independent of any other factors such as lactation, pregnancy or sex. However, it is still controversial whether males and non-pregnant females are susceptible to ketosis [4,9].

Bergmann and Sellers [6] fasted pregnant and non-pregnant guinea pigs for 3 days, during which only the pregnant animals developed clinical signs. Another team fasted non-pregnant females and males for 4 days and provoked subclinical ketosis, but did not report when pathological changes started [4]. Ganaway and Allen [9] were able to evoke a syndrome in obese virgin guinea pigs indistinguishable from pregnancy toxemia. In a study on the influence of vitamin C deficiency on ketosis in young female guinea pigs, the animals were fasted for 10 days and already after 1 to 3 days, an elevation of ketone bodies in the blood could be measured; there was no significant difference between animals with a vitamin C deficient diet and the vitamin C supplemented control group [13].

Apart from anorexia, the affected guinea pigs show clinical signs like reduced activity, ruffled hair, respiratory distress, body weight loss, depression, lethargy, apathy, somnolence, prostration, abortion, stillbirth, convulsions, muscle spasms, paralysis, coma and death [4,6,9,10,12,14]. Ketosis can be diagnosed by blood or urine analysis. In blood serum, measuring the BHB is most sensitive and reflects the progression of the clinical ketosis, as for example BHB represents 80% of the total ketone bodies in cattle [15]. Moreover, BHB is less susceptible to deterioration by storage than acetone and

acetoacetate [16]. In cats, ketone bodies could be detected earlier and in lower concentrations in the blood than in urine [17]. However, there are no published reference values for guinea pigs. In a clinical setting, urine is typically analyzed more often than blood, because sampling is easier and less stressful. Commercially available test strips detect acetoacetate and acetone but not BHB in fresh urine and deliver a semiquantitative result, which should be zero in healthy animals [18].

Further clinical laboratory changes in guinea pigs are acidosis, hyperkalemia, hypocalcemia, hypoglycemia, hyperlipemia, and severely elevated serum cholesterol, as well as ketonuria, proteinuria, aciduria and a decreased urine pH in fasting animals [4,8-10,12,14]. It may not be practical to measure all these values simultaneously due to the amount of blood that can be taken from a live guinea pig, especially not when monitoring changes over time by repeated sampling. Sauer [3] found that fasting ketosis is accompanied by a rapid mobilization of depot fat and therefore an increase of total fatty acids in plasma and concentration in the liver.

Post mortem findings of ketosis typically include significantly more severe fatty livers and hepatic lipidosis of animals fasted after a period of high energy feeding compared to animals fed restrictively [4], and potentially fatty changes in liver, kidneys, adrenal glands and lungs [9]. A retrospective study, based on pathology, reported fatty liver in 72% of guinea pigs with an anamnesis of anorexia [19]. Additionally, of all fatty livers seen, 60% were diagnosed in moderately to highly obese guinea pigs.

Suggested treatments for ketosis include substitutional fluids with dextrose, glucose, calcium and magnesium sulfate as well as nutritional support, if necessary by syringe feeding [7,10,12,14]. Bishop [10] also mentions the use of short-acting corticosteroids as helpful in some cases and recommends monitoring of blood gases, acid/base-ratio, electrolytes, calcium and phosphorus to monitor the progression of the disease. As treatment attempts are often unsuccessful, prevention is considered to be much more important. Factors that should be avoided include obesity, abrupt changes in diet or environment, and other sources of stress. Additionally, an increased supplementation of higher-energy feeds two weeks before parturition (to avoid a reduction of energy intake due to the restricted intake capacity) and encouragement of exercise can be beneficial [12].

The present study was undertaken to gain more detailed information about the etiology, pathogenesis, onset, trend and treatment of ketosis in non-pregnant guinea pigs. Different diagnostic methods were evaluated. First, the possibility to detect ketone bodies in urine of guinea pigs by commercially available urinary dip sticks [a] and its use as an early diagnostic method was assessed. Secondly, a point of care instrument (POC) [b] was tested for its accuracy in measuring BHB in blood. Ketone bodies in urine and blood were measured to investigate any differences in onset, progression and trend as well as the putative synchrony to clinical signs. Additionally, we wanted to test whether a difference between slim and obese animals could be confirmed and whether a therapy had a beneficial effect.

2 Material and Methods

Animals and housing

Fourteen male and female clinically healthy adult guinea pigs each (strain Dunkin Hartley HsdDhl:DH) were used in this study. All the animals were retired breeders from Envigo RMS B.V., The Netherlands, and were at least one year of age. Upon arrival, the animals were divided into four groups (slim and obese females, and slim and obese males) according to their body weight on arrival. All animals were submitted to a general health check with special focus on their teeth to ensure a clinically healthy dentition. The male guinea pigs were castrated to facilitate group husbandry [12]. The study consisted of a feeding and observation period of 59 to 68 days spent in an outside group enclosure, an experimental period of 9 days in individual indoor cages (3 days adaptation, 3 days fasting, 3 days re-feeding/therapy) and again a final observation period in the outside group enclosures of 6 days.

The outside enclosures for each of the two slim groups was 4.45 m in length and 1.12 m in width (approximately 5 m^2). About two thirds of this area was covered by grass, and one third with a substrate of cleaned sand of 1-4 mm in grain size. The enclosure for each of the two obese groups was 2 m in length and 1.12 m in width (2.2 m^2), and the whole area was covered with the sand. Outside enclosures were protected against rain and direct sun. All groups had a variety of shelters at their disposal, whose floors were filled with wood shavings. The individual indoor cages had a ground area of 0.74 m^2 per animal. Opportunity for contact with other individuals was provided by holes in the side walls of the enclosures. The males were kept in one room and the females in another. Every cage had an elevated platform and a shelter. Apple tree branches were offered as gnawing material to all animals in the outside and inside enclosures. Wood shavings were used as litter during the adaptation and treatment period. For the three days of fasting, the litter was changed to sand, to avoid pica behavior.

Feeding

The slim group was fed with grass hay *ad libitum* and the fresh grass that grew in the enclosure. To ensure a steady regrowth of the fresh grass, a certain portion of the grassy area was always fenced off on a rotating basis. The obese group was fed with grass hay (50g/animal and day) and a mixed grain feed [c] for guinea pigs (40g/animal and day). The mixed grain feed had the following ingredients: wheat, oats, barley, corn, peanuts, sunflower seeds, pellets with herbs, vitamins and minerals. Vitamin C substitution was administered by 200 mg ascorbic acid per 1L fresh water [20,21]. Each group had both nipple drinkers and water bowls in the outside enclosure. During single housing, every animal had two nipple drinkers. Water was provided for *ad libitum* intake at all times.

Animal experiment

This experiment was approved by the Animal Care and Use Committee of the Veterinary Office of Zurich (Nr. 27368, ZH003/16). The animals were fed as described above during the feeding period to either keep their slim body condition or to become obese. Animals were weighed once a week. During this period a daily health check was made, consisting of

observing changes in body condition, posture, fur quality, mobility, breathing, group interaction (isolation of group members), external injuries, ocular or nasal discharge, and cleanness of the anal region. Palpation of the abdomen and evaluation of oral and ocular mucosal membrane was performed during weekly weighing. In this period, one slim male animal had an ocular injury (perforated infected corneal ulcer) and had to be treated according to the ophthalmologists' instructions for 14 days. It received Serum-eye drops and Vigamox® [d] (Moxifloxacin) eye drops each 3 to 4 times a day, 0.6ml Metacam® [e] for dogs (Meloxicam, 1mg/kg) and 0.56ml Baytril® [f] 2.5% (Enrofloxacin, 15mg/kg) each per oral once a day and daily probiotic support (Benebac® [f]). This animal later occurred as an outlier in the bile acid measurements and was excluded from statistical evaluation.

One slim male showed a chronic weight loss and did not improve in condition despite additional force feeding with Oxbow's critical care™ [g], and had to be euthanized following the ethical criteria of the study. The necropsy findings were a reduced body weight, diffuse hepatic lipidosis, mild interstitial calcification of the kidney and an alveolar lung edema. No signs of infectious diseases were reported.

The period of individual husbandry including fasting and treatment was done in two batches so that all animals could be evaluated by the same investigator. Due to this sequence, it was decided to first evaluate non-therapy animals in case one of them would develop clinical signs to an extent that required therapy, as requested by the ethical criteria of the study. Because no animal developed clinical illness (see results), this resulted in all animals from the second batch receiving therapy.

During the 9 days in individual cages, the animals were weighed and submitted to a health check in the morning of each day. The start of the 72 hours fasting period was set as time point 0, which is also the beginning of the measurement period. All the substrate, food and chewing material was removed from the cages and a sandy substrate was added instead. Fasting started at 8am. For the refeeding/therapy period, the substrate was changed back to wood shavings and the animals received the normal daily ration of food according to their group (slim/obese). Animals were allocated to a group that was only fed (no therapy), or to a group treated additionally with two subcutaneous injections of Ringer Acetate and Glucose 5% (in a ratio of 50:50) at 20 ml each per day.

At time point 0, samples were collected to determine the basal value for ketone bodies in urine as well as BHB in blood for POC and laboratory analysis. Blood sampling was scheduled subsequently at 72, 84, 96, 108, 120, 132 and 144 hours in all animals, and additionally at 6, 18, 30, 42, and 56 hours in slim and at 12, 24, 36, 48 and 64 hours in obese animals; urine sampling was scheduled for all animals at each of these time points. When urine samples indicated no more ketone bodies an individual animal, only two more subsequent blood samples were taken.

Urine samples were preferably taken from spontaneous urination into transport or anesthesia induction boxes, or otherwise by gentle digital compression on the bladder. Blood samples were taken by venipuncture of alternating sides of the *Vena saphena lateralis* or - under general isoflurane anesthesia induced at 5% isoflurane in an induction box and

maintained at 1.5-2.5% isoflurane (at a mixed air and O_2 flow of 1L/min) by a face mask - from the right or left *V. cava cranialis*, at 0.3 ml per sample. This resulted in a total removal of nearly 4 ml of blood per animal within 144 hours.

For the subsequent observation period, all the animals were returned to their former outside enclosures, in the same groups as before. Their general condition was checked daily for one week.

Sample analysis

The point of care instrument "Freestyle Precision Neo" [h] was used to measure BHB in 1.5 µl of full blood. The test strip contains chemicals that react with BHB and create an electric impulse, which is registered by the instrument and presented on the display after 10 seconds. The POC can read values from 0.0 to 8.0 mmol/L. The laboratory used the BHB LiquiColor® Test [i] to quantify the amount of BHB in serum with an enzymatic approach.

To analyse urine directly, a Combur stick 9® [k] was used. Shortly after collecting the urine, a test strip was dipped into it for approximately 1 second. Approximately 60 seconds later the stick was compared to the colour scale on the container to read the result.

Alanine Aminotransferase (ALT) activity and total bile acids concentration were measured on an automated chemistry analyser [l] using the IFCC method for ALT and an enzymatic method for total bile acids. Two levels of internal quality control samples were measured on a daily basis prior to the patient samples. Furthermore, proficiency testing was performed four times per year.

Termination of the study

The animals were killed by bullet stunning and bleeding. Post mortem blood was collected immediately. A necropsy was performed and organs transferred into 10% neutral-buffered formalin. Liver was taken for histological analysis. The paraffin-embedded tissues were sectioned at 5µm and stained with hematoxylin-eosin (H&E), Periodic acid-Schiff reaction (PAS) and oil-red. A score for liver damage was established and ascribed to each of the slides. Each of the histological liver lobe parts (periportal (1), intermediate (2) and centrilobular (3)) received a separate severity grade from 1 to 3 for degeneration, lipid content, and glycogen content. This led to a maximum score of 27 per animal (3 locations, 3 scores each with a maximum of 3 for each individual score).

Statistical analysis

Data are displayed as means ± standard deviation. Data were analyzed by General Linear Models (GLM; confirming normal distribution of residuals by Kolmogorov-Smirnov-test), with sex, body condition (slim/obese) and, when appropriate, therapy (without/with therapy) as cofactors; if two-way interactions were not significant, the GLM was repeated without the interactions. For liver mass, body mass was added as a covariable in the GLM. If residuals of GLM were not normally distributed, the GLM was performed using ranked data. The scaling of liver mass with body mass was assessed by linear regression of log-transformed values. The risk of hematuria depending on the method of urine sampling was assessed by chi-square test. All analyses were performed in SPSS 23.0 [m,22] with the significance level set to 0.05.

3 Results

Behavioral changes

During the time of fasting, the animals were observed performing coprophagy on a regular basis. They took their feces directly from the anus, but also collected feces off the ground (a behavior termed 'indirect coprophagy'). These observations could not be quantified. Only one animal (number 5), a female belonging to the slim group, showed signs of alopecia because of trichophagia, worsening with time spent individually, and only improving after placing her back into the outside enclosures with other group members.

Body mass

Slim females, arriving with a body mass of 963 ± 45g, did not gain mass during the first observation period (-3.3 ±29.5g), whereas obese females, arriving at 1058 ±29g, gained 86.3 ±61.5g. In males, body mass gains after castration was similar for slim (start 867 ±35g, gain 51.5 ±35.8g) and obese (start 1071 ±71g, gain 45.4 ±52.6g) individuals. At the beginning of the adaptation to the individual cages, the average body mass (±SD) for the individual groups was 972 ±60g for slim and 1140 ±53g for obese females, and 936 ±60g for slim and 1135 ±67g for obese males (Fig. 1).

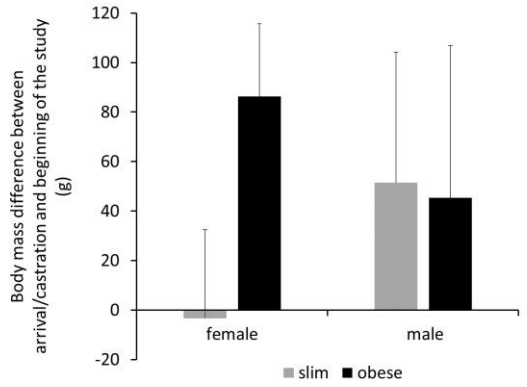

Figure 1. Mean (±SD) difference of body mass (g) from the day of arrival (females) or castration (males) until the beginning of the study period, of all guinea pigs in one group (n=7, except for slim males n=6).

Fasting the guinea pigs for 3 days caused a body mass loss that differed significantly between groups (F = 6.403, P = 0.003), with no differences between the body conditions (F = 1.305, P = 0.265) but lower losses in females (slim 6.9 ±1.6%, obese 7.4 ±1.6%) compared to males (slim 11.3 ±2.9%, obese 9.0 ±1.8%; F = 15.377, P = 0.001). The regaining of body mass within 24 hours differed significantly between the groups (F = 5.206, P = 0.004), with no difference between the sexes (F = 0.706, P = 0.410) and only a trend for a higher mass gain in obese animals (F = 3.840, P = 0.063), a significant effect of therapy (F = 8.275, P = 0.009) and a significant sex x therapy interaction (F = 6.870, P = 0.016), indicating that females gained more mass under therapy than males (Fig. 2).

Two and three days after the termination of fasting, there were no significant differences in body mass gains between the groups.

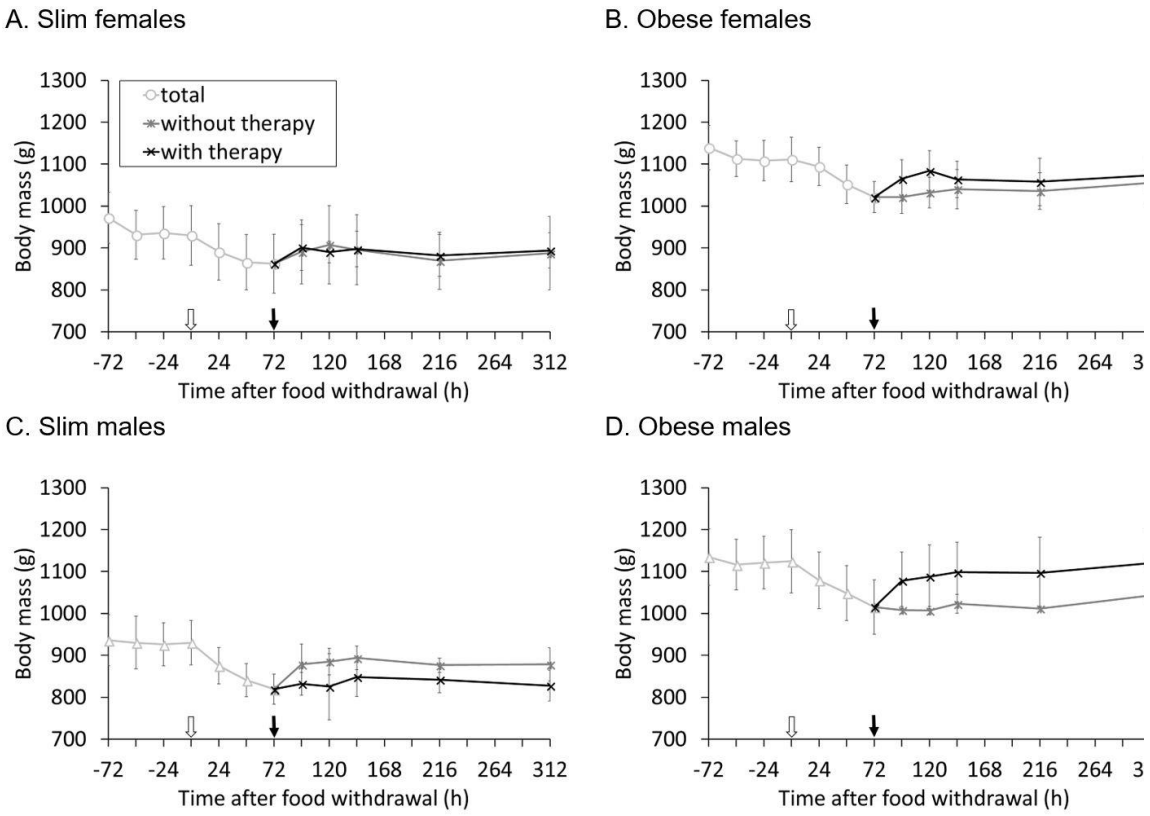

Figure 2. Mean (±SD) body mass (g) of all guinea pigs in one group (n=7, except for slim males n=6); the white and black arrows indicate the beginning and end of the fasting period, respectively. After the end of fasting, animals are divided into therapy groups (n=3/4).

Beta-hydroxybutyrate in blood

The elevation of BHB in serum after 3 days of fasting differed significantly between groups (F = 21.695, P < 0.001) (Fig. 3). Obese guinea pigs had a higher increase than slim ones (F = 52.105, P < 0.001) and females higher than males (F = 5.144, P = 0.033). The interaction sex x obesity showed that there was a greater difference in female guinea pigs between the slim and obese ones, compared to the difference between slim males and obese males (F = 6.970, P = 0.015).

The drop in BHB within the first 12 hours of refeeding also differed significantly between the groups (F = 8.479, P = 0.001), with no effect of sex (F = 0.897, P = 0.354) but a clear effect of body condition (F = 21.305, P < 0.001, obese animals having larger drops) and a trend for a larger drop in animals receiving therapy (F = 3.325, P = 0.082). The drop in BHB within the first 24 hours of refeeding showed the same pattern, with a significant effect of body condition (F = 24.746, P < 0.001) but no effect of therapy (F = 0.204, P = 0.659).

There was a difference between the groups in the time from the end of fasting until the BHB value decreased to normal levels (F = 8.308, P = 0.001). Female guinea pigs needed more hours to normalize their ketone levels than males (F = 12.021, P = 0.002), and so did obese animals compared to slim ones (F = 9.213, P = 0.006). Animals receiving therapy showed a trend to have a shorter recovery time (F = 3.300, P = 0.082).

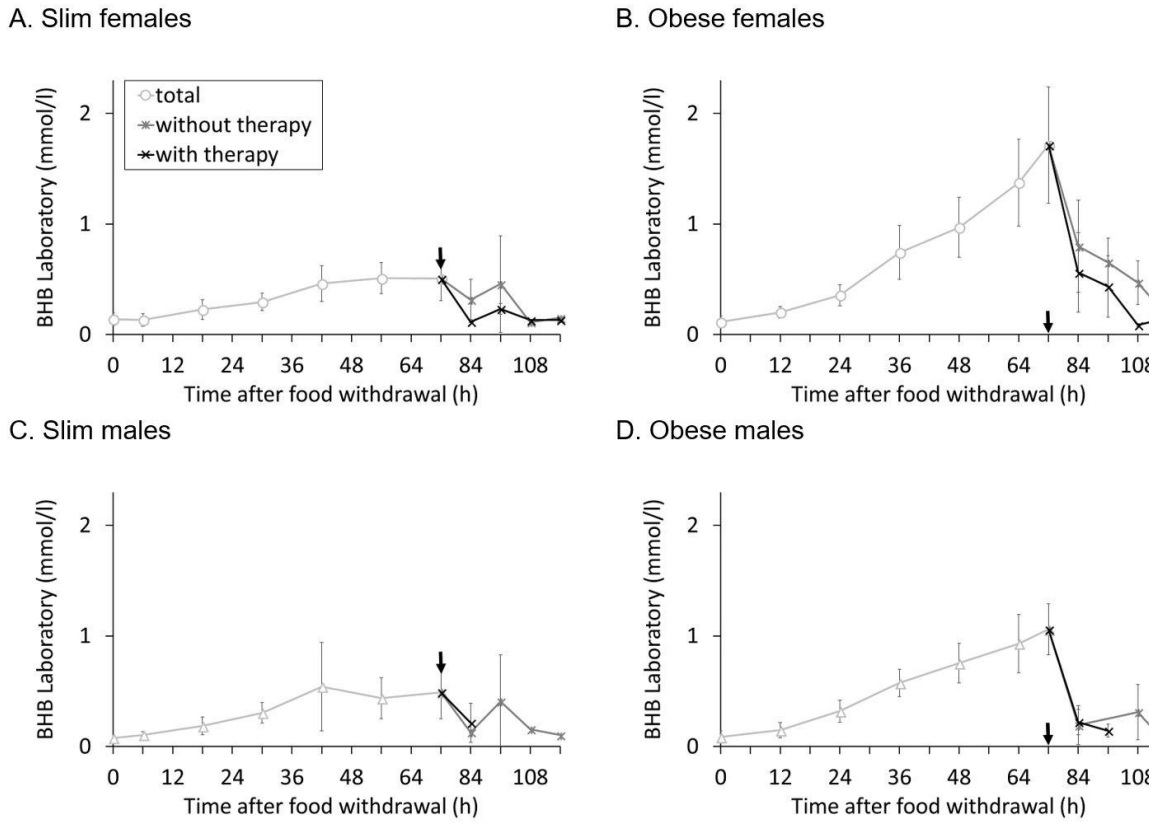

Figure 3. Mean (±SD) β-hydroxybutyrate (mmol/l) in serum (analyzed by the laboratory) of all guinea pigs in one group (n=7, except for slim males n=6); the black arrow indicates the end of the three days fasting period. After the end of fasting, animals are divided into treatment groups (n=3/4).

While POC BHB data showed similar patterns as BHB measured in the laboratory, there was a systematic offset between the two time periods (Fig. 4). Because the data were not normally distributed, not even after log-transformation, a General Linear Model with ranked data was performed, comparing POC data (dependent variable) with laboratory data (independent variable), using sex, slim/obese and therapy as co-factors. Note that in this case, 'therapy' codes for a different time of measurements (9 days difference). The model was significant (F = 68.752, P < 0.001), with a highly significant correlation between laboratory and POC data (F = 153.748, P < 0.001). As expected, neither sex (F = 0.566, P = 0.453) nor obesity status (F = 1.899, P = 0.170) had a significant effect on the relationship. However, the time of the experiment, coded by therapy, had highly significant influence (F = 92.855, P < 0.001).

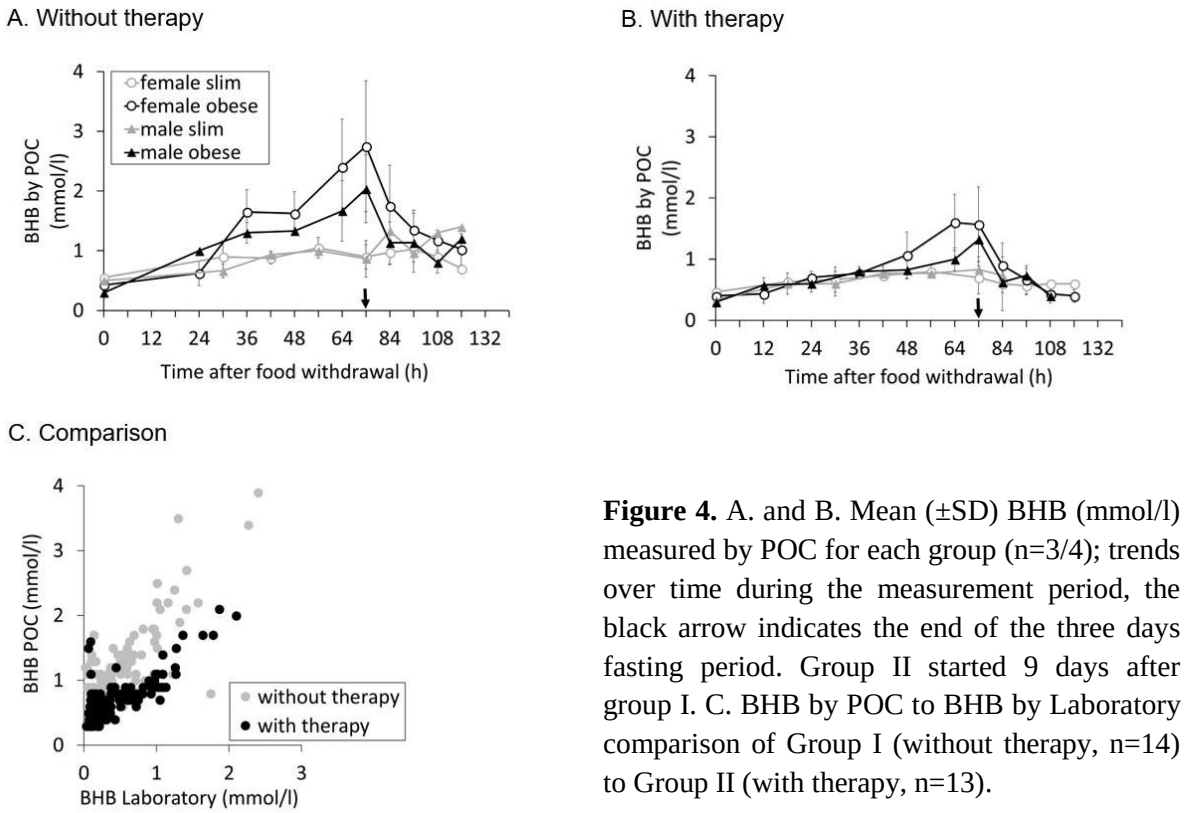

Figure 4. A. and B. Mean (±SD) BHB (mmol/l) measured by POC for each group (n=3/4); trends over time during the measurement period, the black arrow indicates the end of the three days fasting period. Group II started 9 days after group I. C. BHB by POC to BHB by Laboratory comparison of Group I (without therapy, n=14) to Group II (with therapy, n=13).

Ketone bodies in urine

Ketone body levels in urine increased during the fasting period, with significant differences between all groups (F = 12.363, P < 0.001) (Fig. 5). Obese guinea pigs showed more intense ketonuria than slim ones (F = 19.664, P < 0.001) and females more than males (F = 5.850, P = 0.024). The time from the end of fasting to the normalization of urinary ketone body levels (i.e., levels of 0) also differed significantly between the groups (F = 9.874, P < 0.001), with obese animals requiring more time to normalize their ketonuria in comparison to the slim ones (F = 12.701, P = 0.002), females compared to males (F = 10.152, P = 0.004) and animals not receiving therapy compared to animals receiving therapy (F = 6.307, P = 0.019).

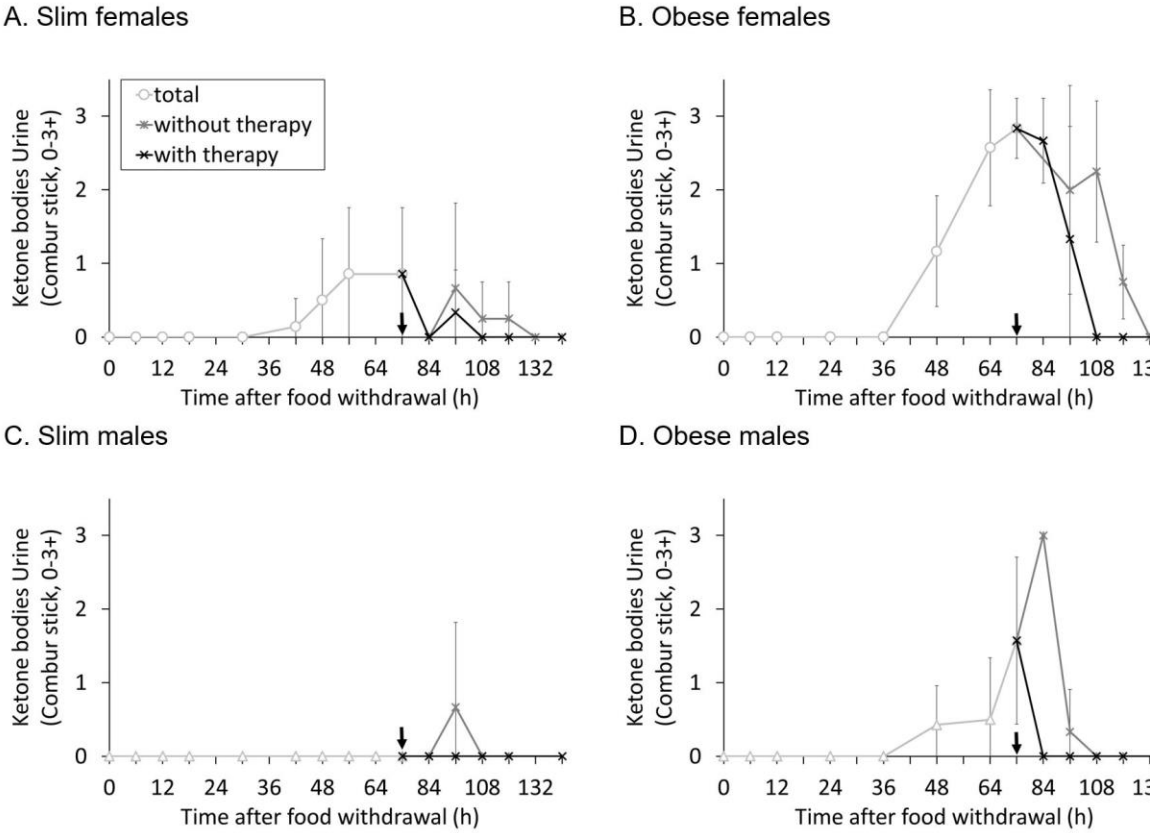

Figure 5. Mean (±SD) ketone bodies (semiquantitative, 0-3) detected in urine with Combur stick 9© of all guinea pigs in one group (n=7, except for slim males n=6); the black arrow indicates the end of the three days fasting period. After the end of fasting, animals are divided into treatment groups (n=3/4).

Hematuria

There was significantly more hematuria detected by urinary sticks in samples produced by digital pressure on the bladder than in samples produced spontaneously (chi-square = 6.514, $P = 0.011$).

Liver to body mass

Liver mass was significantly related to body mass (F=22.389, P<0.001), with no effect of sex (F=1.291, P=0.268), body condition (F=2.056, P=0.166), or therapy (F=0.004, P=0.953). Liver mass scaled [with confidence intervals] to 0.0003 [0;0.0029] $BM^{1.66[1.32;1.99]}$. When assessing slim and obese animals separately, the corresponding equation was 0.0150 [0;11.4025] $BM^{1.07[0.09;2.05]}$ for slim and 0.0009 [0;2.2542] $BM^{1.50[0.38;2.62]}$ for obese animals (Fig. 6).

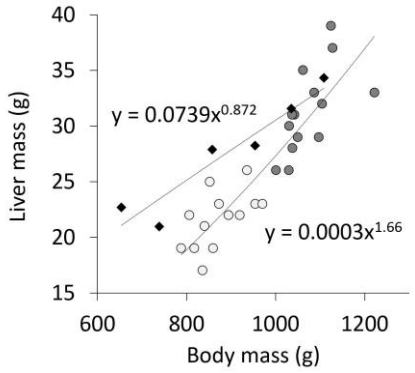

Figure 6. Liver mass (g) in relation to body mass (g) of guinea pigs at time of necropsy after decapitation and bleeding out; our study (grey and white dots) compared to the study by Webster and Liljegren [37], 1947 (black squares), and the corresponding trendline.

Liver histology and laboratory values

The lipidosis score was not affected by sex, body condition, or therapy; only the sex x therapy interaction was significant (Fig. 7A; for statistics of all histology and laboratory values see Table I). The degeneration score was not affected by sex, but strongly affected by body condition and by therapy, with a significant therapy x body condition interaction (Fig. 7B, Table I). More degeneration was seen in obese compared to slim guinea pigs and in therapied versus non-therapied animals. The interaction represents an effect of therapy in obese animals, showing more degeneration with therapy; this was not seen in slim animals. The same significances were seen for glycogen content score, with an additional trend for sex, indicating a higher glycogen content in males than in females (Fig. 7C, Table I). The interaction confirmed a higher glycogen content in livers of therapied obese animals compared to non-therapied obese animals. The same pattern was seen in slim animals, but was not as distinct. The total liver damage score was only affected by body condition, indicating more liver damage in obese animals (Table I).

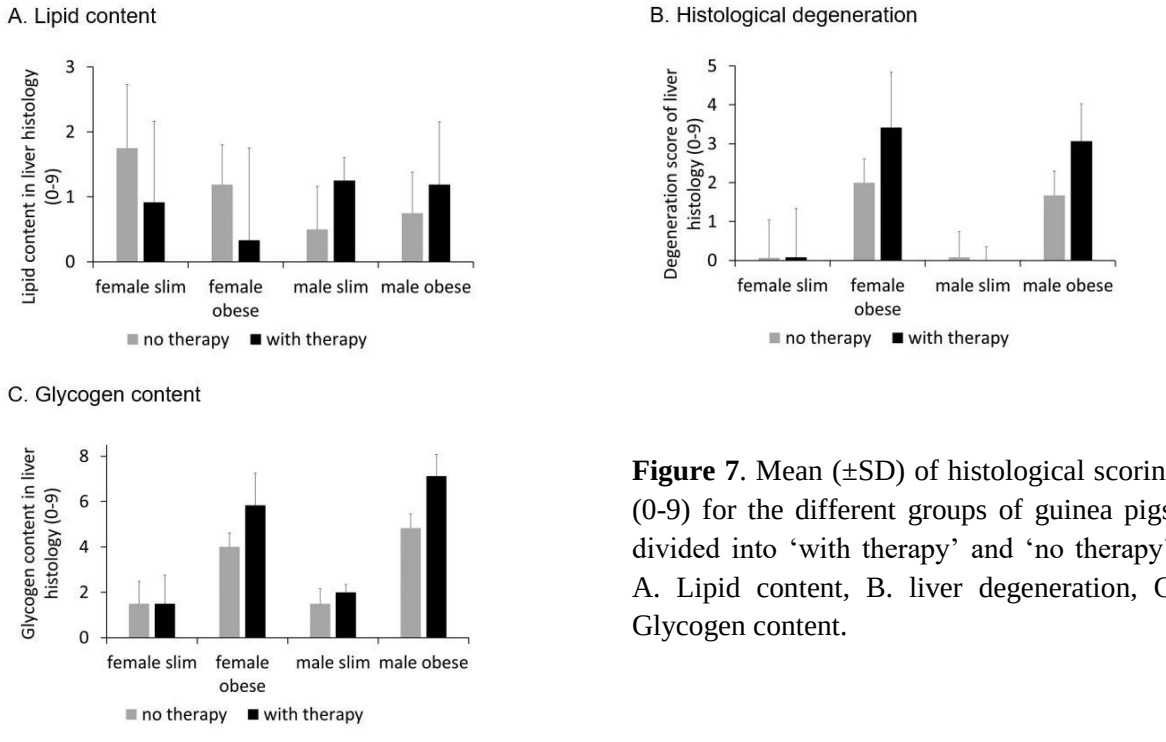

Figure 7. Mean (±SD) of histological scoring (0-9) for the different groups of guinea pigs, divided into 'with therapy' and 'no therapy'. A. Lipid content, B. liver degeneration, C. Glycogen content.

The ALT values showed higher results in therapied animals; however, all but two animals were within the reference range (Table I). Bile acids were highly affected by body condition and therapy, with a significant interaction of sex x therapy. They showed higher values in obese and in therapied animals (Table I).

Table I. Statistical data of liver histology scores and laboratory values, comparison between the different groups of guinea pigs. (*****ranked data)

	Sex	Body condition	Therapy	Interaction
	F (*P*)			
Lipidosis score	0.121 (0.731)	0.583 (0.453)	0.121 (0.731)	sex x therapy: **4.570 (0.044)**
Degeneration score *	2.031 (0.168)	**137.296 (<0.001)**	**4.619 (0.043)**	therapy x body condition: **7.427 (0.012)**
Glycogen content score	4.273 (0.051)	**117.924 (<0.001)**	**12.301 (0.002)**	Therapy x body condition: **6.698 (0.017)**
Total Liver damage score*	0.826 (0.373)	**34.760 (<0.001)**	2.053 (0.165)	-
Bile acids	2.981 (0.099)	**5.366 (0.031)**	**10.646 (0.004)**	sex x therapy: **7.885 (0.011)**
ALT*	2.222 (0.150)	1.379 (0.252)	**5.186 (0.032)**	-

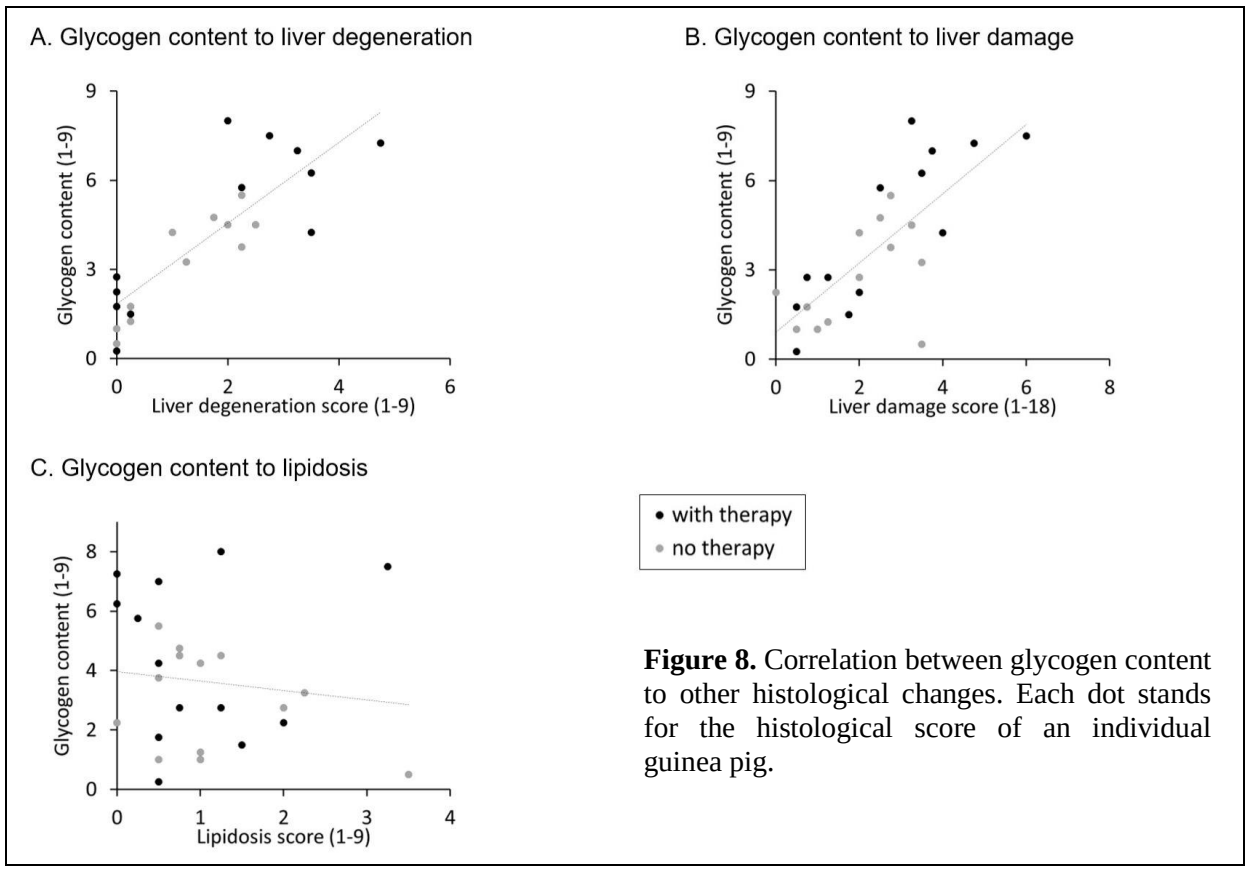

Figure 8. Correlation between glycogen content to other histological changes. Each dot stands for the histological score of an individual guinea pig.

Additionally, there was a significant correlation between the glycogen content and the liver degeneration score (ρ = 0.83, P < 0.001, n = 26) and the liver damage score (ρ = 0.71, P < 0.001, n = 26) (Fig. 8A+B), as well as between bile acids and the liver degeneration score (ρ = 0.44, P = 0.026, n = 26) and the liver damage score (ρ = 0.59, P = 0.002, n = 26) (Fig. 9A+B). In contrast, the liver enzyme ALT did not correlate with the liver damage score (ρ = -0.06, P = 0.790, n = 26) (Fig. 9C), and neither did the score of lipidosis to glycogen content (ρ = -0.20, P = 0.337, n = 26) (Fig. 8C).

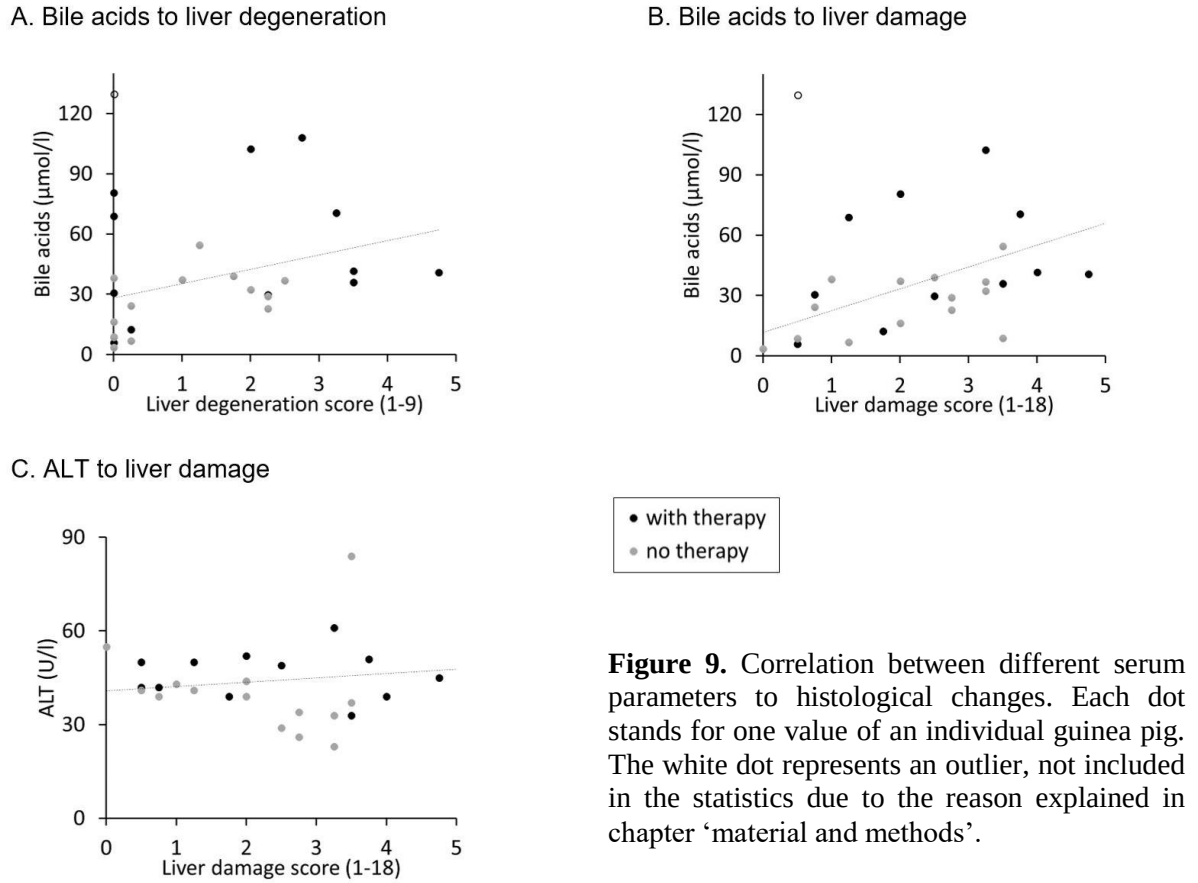

Figure 9. Correlation between different serum parameters to histological changes. Each dot stands for one value of an individual guinea pig. The white dot represents an outlier, not included in the statistics due to the reason explained in chapter ‘material and methods’.

4 Discussion

Our study focused on the development of fasting ketosis in guinea pigs, predisposing factors, diagnostic tools and therapy attempts. We corroborated that female individuals seem to be more severely affected by ketosis, as well as obesity as a predisposing factor for this metabolic disorder, and for longer recovery times thereafter. A positive effect of our therapy protocol with glucose could be demonstrated through improving several clinical indicators of recovery, but it also caused hepatocyte pathology. An additional finding was the evaluation of the POC instrument “Freestyle Precision Neo” for guinea pigs. The trend of POC values showed a good relationship to the laboratory data, although the accuracy of the exact values was weak. However, an accurate validation that follows the ASCVP-guidelines would need more than just a comparison between two tests.

During the time of sample collection, hematuria was documented in multiple cases. Further analyzing our data displayed a higher degree in hematuria when urine samples were collected through digital pressure on the bladder, supporting findings in previous studies.

Generally, our study only provoked a subclinical ketosis. A stronger reaction, with more distinct differences between groups, would have required a more prolonged fasting period.

Behavioral changes

Coprophagy is a normal behavior performed by several small mammals including guinea pigs. The ingested feces had no changes in appearance to normal excrement. Both behaviors, direct and indirect coprophagy, were formerly described [23]. Alopecia as a cause of trichophagia is a known issue if nutritional supply is quantitatively or qualitatively unsatisfying [24,25]. Interestingly, only one animal in our study showed this behavior, even though all animals were fasted for three days and a higher prevalence had been expected.

Hematuria

Urine collection through digital pressure on the bladder led to more hematuria than collecting spontaneous urinary samples. Nevertheless, 30% of the animals with spontaneous urination showed amounts of blood in urine, and half of the animals where the bladder was emptied through manipulation did not show signs of hematuria. Note that the Combur© stick does not differentiate between Hemoglobin and Myoglobin. The hematuria can be explained by traumatic microlesions in the urinary tract caused by the forced emptying of the bladder. Our findings are in agreement to formerly observed urinalysis, where less blood was seen with spontaneous urination [18].

BHB by POC

The accuracy of the POC in our study was less precise than reported in other studies for various animal species. In a recent meta-analysis of 18 studies on the diagnostic accuracy of POC instruments for the detection of ketone bodies, an excellent accuracy of Precision Xtra™ for the use in cattle was reported [26]. Additionally, in a fact sheet by Oetzel and McGuirk [27] it was suggested to set the threshold for diagnosis of ketosis with POC values a little lower, because the hand-held ketone meter gave slightly lower test results than the laboratory. Another POC instrument, Precision Xceed® by Abbott® was validated with studies on sheep and cats, showing close correlation with the laboratory reference method [28,29]. In dogs, an overestimation of BHB concentrations by POC measurement was seen; however, a positive correlation to the laboratory values led to the conclusion that this POC was a useful tool in assessing ketonemia [30].

Our study shows a similarity between the measurements by the POC and the laboratory, but altogether higher values resulted from the POC. Additionally, even higher values that show a greater deviation from the laboratory values were documented for the group without therapy. A difference of nine days lay between the two groups of therapy, due to logistic reasons explained above, and great effort was put into establishing the same environmental conditions: room temperature fluctuated only around ± 2°C with a similar average temperature in both groups. Regrettably, no calibration of the

instrument was made ahead of the measurement period, as it was not considered necessary by the manufacturer; this might be considered as a cause of the inaccuracy. Nevertheless, we find that the increase and decrease of BHB can be displayed adequately by using the POC, which makes it a suitable tool to interpret a trend. For a single time point value, BHB as determined by laboratory methods appear as the safer option.

Body mass

Surprisingly, only the obese females gained a substantial amount of body mass (86.3g ±61.5) during the feeding period, whereas the slim females just barely kept theirs. In contrast, both male groups gained a similar amount (45.4g ±52.6 and 51.5g ±35.8), which was roughly half of the obese females' body mass gain. Considering the impact of castration and the stressful condition prior to this, one could argue that the males only regained their body mass already lost for these reasons. Nonetheless, we expected a greater increase in body mass of high energy-fed animals, as seen in Lachmann, et al. [4]. Pitts [31] found that female guinea pigs have a greater capacity to store fat in comparison to males, which could explain the difference seen between the obese females and males in our study.

The body mass loss of 6.9 ±1.6% to 11.3 ±2.9% after 3 days of fasting in our study is less than documented in former studies, where losses of 12 to 25.5 ±1.8% within 3 to 4 days were seen [4,6,9,32]. A great proportion of the body mass loss during fasting is presumably the loss of ingesta from the digestive tract as discussed in Bergmann and Sellers [6]. Our guinea pigs were observed to perform coprophagy on a regular basis, which could explain the less severe body mass loss. Additionally, most of the former studies were performed by using young animals, still in growth and therefore of lesser body mass to start with, which could have led to a greater impact of starvation.

Females gained more mass than males in the pre-experimental phase, yet the males still lost more during fasting. Within the first 24 hours after refeeding, a trend in greater mass gain of obese animals was seen. In our study the guinea pigs were initially separated into groups according to their arrival body mass and thereby possibly also selected by their tendency to gain mass, determined by genetic or epigenetic factors. Additionally, female guinea pigs under therapy gained significantly more weight on day 1 compared to the therapied male group. However, after day 2 no difference was seen anymore.

Development and regression of ketosis

According to Kraft, et al. [15], healthy animals do not excrete any ketone bodies in urine and their blood level of BHB is less than 0.6 mmol/l. In cows, the threshold for subclinical ketosis is set at 0.9-1.7 mmol/l BHB in serum; for a value above 1.7 mmol/l BHB in serum, clinical manifestation is to be expected. Looking at our values, the threshold might be similar. Yet, we cannot define a threshold for subclinical to clinical ketosis, as we did not induce any clinical signs through our study settings. Additionally, pregnant guinea pigs might be more susceptible and have a lower threshold, because in Lachmann, et al. [4] obese pregnant females had lower average BHB values and became severely ill, likewise in Ganaway and Allen [9] where non-pregnant obese female guinea pigs showed signs of ketosis but not as severely

as pregnant ones. Probably, more time is needed until animals become clinically ill: In Lachmann, et al. [4] nonpregnant females and males started to show signs of illness after 4 days of fasting. Further, the different onset of a clinical disease could be explained as follows: If animals develop ketosis as a secondary problem, they might be weak already, and might not perform coprophagy that could delay the process. However, this hypothesis requires further investigation.

As suspected, obese guinea pigs showed a higher susceptibility to develop a metabolic imbalance while fasting compared to slim ones. Over a period of 3 days' fasting, the elevation of BHB in serum showed significantly higher values in obese guinea pigs and a significantly more intense ketonuria. After refeeding, the animals with an adipose body condition needed more time until BHB values decreased to normal levels and ketone bodies were eliminated from urine. A study by Ganaway and Allen [9] also induced higher serum BHB in adipose animals and reported an outcome in non-pregnant guinea pigs after fasting similar to ours. This outcome is explainable by the greater fat storage of high energy fed animals prior to fasting. Therefore, more fat is mobilized in an anorexic stage and transported to the hepatocytes, where an excessive supply leads to ketone body production and hepatic lipidosis [4,6,8,19]. Additionally, the impact of insulin should be discussed, as it has an antilipolytic effect. Obese animals can form an insulin resistance, leading to higher lipolysis and consequently more ketogenesis and lipidosis than in leaner individuals [33,34].

Fasting seems to have a greater impact on female animals than males, expressed through the higher BHB serum values and more ketone bodies detected in urine of females. In our study, a greater difference in females between the obese and the slim group compared to the equivalent male groups was seen. Our findings agree with Butts and Deuel Jr [35], who found that female guinea pigs excreted twice the amount of acetone bodies than their male counterpart after administration of acetoacetic acid. They relate this disparity to sexual difference in the ability to oxidize acetoacetic acid and claim a higher susceptibility to ketosis for female individuals. No blood parameters were measured in that study. In contrast, the study by Lachmann, et al. [4] found male guinea pigs to excrete more ketone bodies in urine and form higher BHB peak values than females after 4 days of fasting (Table II). However, only acetone in urine was measured in their study, whereas acetone and acetoacetate were measured in our experiment. Also, the male guinea pigs of Lachmann, et al. [4] were heavier (+173 g) prior to the fasting period, due to the greater mass gain through high energy feeding, and were perhaps more obese than the females. Possibly obesity is the decisive factor, and differences between studies could then be explained through the difference in body mass of the male and female individuals.

Table II. Comparison of serum BHB values after fasting guinea pigs for 3 and 4 days from two different studies

	Lachmann et al. 1989 (4 days fasting)			**our study (3 days fasting)**		
	BHB value (mmol/l)	Body mass (g)	number of animals	BHB value (mmol/l)	Body mass (g)	number of animals
female obese	0.83 ±0.69	855 ±131	6	1.71 ±0.53	1145 ±56	7
male obese	1.40 ±0.39	1030 ±175	14	1.06 ±0.23	1117 ±71	7

After the end of the fasting period, females required more time to normalize their BHB levels in serum and to cease their ketone body excretion in urine. Bacchus, et al. [13] injected BHB intraperitoneally into young female guinea pigs, determined the total ketone body concentration in blood through measuring acetone, and reported a half-life time of 68 (±2.1) min. Considering the final BHB values at the end of the fasting period in our study and the next subsequent BHB measurement after 12 h, the theoretical half-life time of our values is, in contrast, about 6 to 12 hours. We are not able to define an accurate half-life time, because our measurement interval was not as frequent. However, the difference seems reasonable, as the animals in the prior study were healthy and only had to eliminate the injected BHB, whereas our animals produced BHB by themselves, and had to down-regulate production in parallel to eliminating the product.

Effects of therapy on the regression of ketosis

Positive effects of therapy versus no therapy, i.e. additional glucose injection in contrast to merely refeeding, were observed. First, a trend for larger drops in BHB levels within the first 12 hours after fasting were seen. Secondly, those animals receiving therapy tended to have a shorter recovery time considering BHB level decrease, as well as urine ketone body elimination. Studies in rats on fasting ketosis by Foster [36] described an abrupt cessation of ketone body production by the liver after intravenous administration of 0.3 ml glucose 50%, and a decline of acetoacetate began within 5 minutes. Moreover, tube feeding of 5 ml high glucose diet led to a reversal of ketosis within 15 min, inducing the same metabolic effect. This is similar to our findings, but much faster and explained by the more intense intervention in those experiments.

Comparison of urine to serum ketone body remission

Our hypothesis was that ketone bodies in blood would disappear earlier than in urine, which we could not confirm. Ketone bodies detected by urinary dip stick are only acetoacetate and acetone, but the greatest fraction of ketone bodies in fasting ketosis is usually BHB at 80%, and a change in color of the urine test stick is only detectable when ketone bodies exceed a certain concentration in urine [15]. However, in the ketone body cascade in direction of ketone body reduction, BHB is metabolized to acetoacetate and further to acetyl-CoA, which is being integrated into the citric acid circle if enough oxaloacetate is available, or alternatively reversed to the fat storage as triglycerides. This would mean that urinary dip sticks do not

represent the full extent of the disease in the fasting stage. Considering the ketone body cascade, the assumption can be made that acetoacetate degrades as the latest of all ketone bodies and is a good indicator of ketosis remission.

Liver mass to body mass

The liver mass in comparison to body mass has an unusual scaling of $y=0.0003x^{1.66}$. Normal liver mass to body mass was described by Webster and Liljegren [37], where they measured different organs of guinea pigs. The trendline of their values shows a gradient of $y=0.0739x^{0.87}$ (Fig.6). This matches the statement by Rocha, et al. [38] that liver mass is aligned with the overall organism's metabolism. Our result clearly deviates from these findings. The exponent found by Webster and Liljegren [37] was included in the 95% confidence interval of the slim animals; even though the scaling exponent for liver mass did not differ significantly between slim and obese animals in our study (due to overlapping 95% confidence intervals), the scaling was steeper in the obese specimens, suggesting that the overall extreme scaling in our animals derived from a pathological condition of the liver due to fasting that was particularly pronounced in this group. Therefore, the liver was evaluated histologically.

Histological findings of the liver

Guinea pigs suffering from ketosis showed very fatty livers at necropsy, and the livers were 10% heavier than the ones of control animals [6]. In our study, no significant difference of lipidosis between the obese and the slim guinea pigs was documented. Females receiving therapy showed less hepatic lipidosis than the ones not treated, and the opposite outcome was seen in males, where the treated animals showed more signs of lipidosis. Several other studies found severe fatty livers in obese guinea pigs following an anorexic period, reporting higher lipid content in the liver of obese animals versus those of a normal body condition and more in livers of ketonic guinea pigs than healthy ones [3,4,8,19,39-41]. These findings were all documented directly after the fasting period, when highest fat mobilization was in progress. Evaluation of the livers in our study took place one week after refeeding, probably explaining the difference in outcome. Nevertheless, obese animals probably mobilized more fat to the liver while fasting, and therefore more hepatic degeneration was seen in obese animals compared to slim ones at the end of the study.

A trend was seen for higher glycogen content in livers of males in comparison to females, confirming previous findings [42], although no higher lipid content was seen in female livers in our study. Foster [36] described a negative correlation between the lipid and glycogen content of the liver, with glycogen declining while fat content increased during fasting. Similar findings are shown in Bergman and Sellers [6]. No correlation was seen at the timepoint of our measurement, and no trend over time was recorded. Nevertheless, we saw obese guinea pigs to have significantly higher glycogen contents in the liver in comparison to slim animals, probably due to the different diet received. The obese group on an energy-dense diet was supplied with hay and a mixed grain feed *ad libitum* whereas the slim groups only had hay and grass at their disposal. Fréminet [32] described hepatic glycogen content in rats and guinea pigs to be almost exhausted within 24 hours of food

deprivation and remaining low until 96 hours of fasting; after refeeding, the hepatic glycogen content exceeded the one of the control animals.

Another predictable difference was between therapied and non-therapied guinea pigs. Those having had fluid and glucose injection showed more glycogen in their liver. The injected glucose is primarily oxidized directly for energy, and the remaining glucose in depleted animals transforms predominantly toward hepatic glycogen [43]. Additionally, we revealed a correlation between glycogen content and damage of the liver. As described in Fréminet [32] depleted animals are more likely to store additional glucose as hepatic glycogen. Excessive glucose substitution could have led to a greater impact on the liver through a glucose overload, with the following storage as hepatic glycogen and consequently a delay of regeneration from hepatic lipidosis. This might be an explanation for the greater hepatocyte degeneration seen in therapied guinea pigs. Probably, an initial dose of glucose to stop ketogenesis is beneficial. Afterwards, the animal should be observed carefully, and glucose therapy only continued if the animal does not start eating on its own.

Laboratory parameters

In vivo testing of serum parameters to evaluate liver alterations is a less invasive method than taking biopsies for histology. Therefore, ALT and bile acids were measured in our study to evaluate their use in guinea pigs. ALT is relatively specific to liver in rats and an accepted biomarker for the detection of liver injury in preclinical models [44-46]. However, this liver enzyme is not convincingly associated with histopathological findings [47,48]. This stands in agreement with our findings: No values outside the reference range were found and no correlation to the liver damage score documented, which makes ALT a non-reliable parameter for liver injury in guinea pigs. In contrast to this, bile acids showed a significant correlation to the liver damage score. Higher bile acid levels were seen in obese animals, reflecting our overall finding of obese guinea pigs being more severely affected by the impact of fasting. Bile acids were described in various species of having a high association with liver diseases, hepatic damage or fatty liver [48-51]. A drawback of the total bile acids is that they are only a sensitive indicator for an overall assessment of hepatic damage but give little insight in the specific damage or pathogenesis. The changes in bile acids suggest a decrease in liver function; therefore, it would be interesting to see whether other liver function parameters such as total protein, fibrinogen, urea and clotting factors are abnormal, too. Additionally, it might be worthwhile testing the use of urine for bile acid screening [52] in guinea pigs as they are an easily stressed species if handled too intensively.

Conclusion

An anorexic state in guinea pigs should be considered as a serious condition, likely leading to death if initiation of treatment fails. It is therefore essential that those cases are treated immediately to reverse the katabolic state they are usually in at the time of presentation. Supportive fluid therapy with glucose supplementation is helpful in this case, as it terminates the production of ketone bodies and reduces the metabolic imbalance. As an additional benefit, we saw that resolution of the metabolic disorder seems faster when

given supportive therapy. To follow the trend of ketosis and the change in metabolic condition of the animal the POC tested presents itself as a valuable tool to detect trends, however not as an appropriate indicator of the exact values. Measuring the bile acids seems a helpful value to detect an impact on the liver and to estimate histological damage. Further studies which create a more intense metabolic imbalance through fasting for longer time periods are suggested to investigate clinical manifestations of ketosis. As an addition, liver values should also be measured during the fasting and refeeding period instead of at the end of the experiment; this would ensure a more accurate evaluation of liver parameters.

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Footnotes

[a] Combur 9©, Roche Diagnostics GmbH, D-68305 Mannheim, Germany

[b] FreeStyle Precision, Abbott Diabetes Care Ltd, Oxon OX29 OYL, UK

[c] vita-balance, LANDI Art. 26267, Landi, CITY, Switzerland

[d] Novartis Pharma AG, 6343 Rotkreuz, Switzerland

[e] Boehringer Ingelheim GmbH, 4002 Basel, Switzerland

[f] Provet AG, 3421 Lyssach, Switzerland

[g] Oxbow Animal Health, Omaha, NE 68138 USA

[h] Abbott© Diabetes Care Ltd, Oxon OX29 OYL, UK

[i] interchim®, 03103 Montlaçon Cedex, France

[k] Roche Diagnostics GmbH, D-68305 Mannheim, Germany

[l] Cobas 6000 501, Roche Diagnostics, Rotkreuz, Switzerland

[m] IBM, Armonk, New York, USA

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